FUNCTION OF TWIST1 TRANSCRIPTION FACTOR IN AORTIC VALVE CALCIFICATION

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN
BIOTECHNOLOGY

Submitted By

Somdutta Bhattacharyya

Registration ID: 230020353375

Enrolment ID:2322210002035

Advisor

Dr. Santanu Chakraborty

Designation: Assistant Professor of the Department of Life Science

Affiliation: Presidency University

School of Health Science and Translational Research
Sister Nivedita University
Kolkata

June 2025

Presidency University

Hindoo College (1817-1855), Presidency College (1855-2010)

TO WHOM IT MAY CONCERN

This is to certify that **Somdutta Bhattacharyya**, a M.Sc. student of the Department of Biotechnology, Sister Nivedita University, Kolkata has successfully completed her internship/dissertation research work in my research laboratory for the duration of about five months (**02/01/2025 to 30/05/2025**). During her tenure she got the opportunity to work on several molecular and cell biology techniques including but not limited to primary animal cell culture of isolated valvular interstitial cells isolated from adult porcine heart, immunocytochemistry/immunohistochemistry, protein/RNA isolation, *in vitro* inhibitor/activator studies, concentration measurements (spectroscopy), Real time PCR, Western blot, Histochemical staining (Von-Kossa and Alizarin red) and imaging (Fluorescence microscopy).

In closing, I certify that Somdutta is a very punctual, obedient, inquisitive, dedicated, hardworking and sincere in nature. She is very attentive and regular and tries to give her best to assigned tasks. Apart from being a hard worker and sincere student, she has a habit of keeping herself up to date in relevant literature. I wish her success for all future endeavors in her life and career.

Best Regards,

Dated: June 02, 2025

Santanu Chakraborty, PhD
Assistant Professor (Level III),
Department of Life Sciences,
Presidency University, Kolkata

86/1,Collegestreet,Kolkata-700073,Phone:(033)22411960,Fax:(033)22412738

DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**Function of Twist1 transcription factor in Aortic Valve Calcification**" in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology is an authentic record of my own work carried out during the period from January 2025 to May 2025 under the supervision of Dr. Santanu Chakraborty Department of Life Sciences.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

I confirm that this dissertation has been checked for plagiarism using the plagiarism checking

Software Turnitin. At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed. I herewith confirm that the dissertation has been less than 10% similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University.

I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations.

Somdutta Bhattacharyya
04.06.2025

(Signature of the students with Date)

Name of the Student – Somdutta Bhattacharyya

Registration no - 230020353375

Department-Biotechnolog

SISTER NIVEDITA UNIVERSITY
সিস্টার নিবেদিতা বিশ্ববিদ্যালয়

DG Block - 1/2, Action Area I, New Town, Kolkata - 700156, West Bengal, India.

DEPARTMENT OF BIOTECHNOLOGY & DEPARTMENT OF MICROBIOLOGY
জৈব প্রযুক্তি বিভাগ ও অণুজীব বিদ্যা বিভাগ

TO WHOM IT MAY CONCERN

This is to certify that the project report titled “Function of Twist1 transcription factor in Aortic Valve Calcification", submitted by Ms. Somdutta Bhattacharyya (Registration No. 230020353375, Enrolment no 2322210002035) to the Department of Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 03.06.2025

Place: SISTER NIVEDITA UNIVERSITY

Signature of Head of the Department

Seal

Head
Department of Biotechnology
Sister Nivedita University
Block DG-1/2, Action Area 1
New Town, Kolkata-700 156

Acknowledgment

From the bottom of my heart, I would like to express my sincere gratitude to my supervisor, Dr. Santanu Chakraborty, Department of Life Sciences, University of Presidency, for his constant encouragement and guidance throughout my dissertation project, which has inspired me and motivated me a lot in the completion of this project work. I am fortunate to have him as my supervisor.

I express my sincere gratitude to Dr. Somsubhra Thakur Choudhury, Head of the Department of Biotechnology, Sister Nivedita University, who allowed me to undertake this dissertation project. And to all the Faculty members of the department for their encouragement.

I am also thankful to my research scholar, Miss. Madhurima Ghosh, for her valuable input and guidance throughout my dissertation. I would also like to thank my lab mates Sneha Roy, Bidipta Das, and Subhadip Dey, and other research scholars, Mr. Pabitra Mondal, Mr. Ipshit Dey, who worked alongside me in the lab. I would finally like to thank all my friends and family for their continuous support and inspiration during this journey. They have been a continual source of inspiration and comfort through both difficult and rewarding times because they love me, understand me, and believe in my skills and potential.

List of figures

Figure no	Title	Page no
1	Porcine whole heart and aortic valve	12
2	Von Kossa staining and Alizarin staining	12
3	Result analysis of qRT-PCR of Bmp2, OCN and OPN	13-14
4	Result of Immunofluorescence staining of Twist1 and α-SMA	15
5	Result of Immunofluorescence staining of Twist1 and E protein	17
6	Result of Immunofluorescence staining of Id1 and E protein	18-19

Abbreviations

CAVD - Calcific aortic valve disease
bHLH- Basic helix-loop-helix
GAGs - Glycosaminoglycan
ECM - Extracellular matrix
VICs- Valvular interstitial cells
VECs- Valvular endothelial cells
LDLs- Low-density lipoproteins
EC- Endocardial cushion
PBS- Phosphate buffer saline
DMEM- Dulbecco's Modified Eagle Medium
FBS - Foetal bovine serum
BSL2- Biosafety level 2
PCR - Polymerase chain reaction
TGF-β - Transforming growth factor-
OCN - Osteocalcin
OPN - Osteopontin
BMP2- Bone morphogenetic protein 2
α-SMA- Alpha smooth muscle actin
Id1- Inhibitor of differentiation1
Tcf3- Transcription factor 3

Abstract

The gradual and persistent illness known as calcific aortic valve disease (CAVD) can range from aortic sclerosis, which is a minor thickening of the valve without blood flow restriction, to severe calcification that limits leaflet mobility, referred to as aortic stenosis. Osteoblasts in CAVD suggest that calcification is a dynamic and regulated phenomenon characterised by lipoprotein accumulation and persistent inflammation. In diseased human valves and various animal models, such as porcine and mouse, there is an induction of valve progenitor markers, including Twist1 and Tbx20. Further indicators of bone formation, such as Runx2, Bmp2, osteocalcin (OCN), and Osteopontin (OPN) are likewise present in calcific aortic valve disease (CAVD). Twist1, a class II basic-helix-loop-helix (bHLH) transcription factor, is expressed in the early stages of endothelial cell development and subsequently shows reduced expression during the remodelling of valves. In aortic valves affected by disease, there is an observed increase in Twist1 expression and cell proliferation near the calcification nodules. This study was conducted to explore the detection of calcification within tissues and cells, identify the downregulation and upregulation of genes and markers associated with calcification, visualise the localisation and concentrations of calcium salts, and assess protein dimerization. Given that the differentiation of valve osteogenic cells resembles physiological osteogenesis, we proposed that the dimerization of twist and E proteins, which occurs during skull development, also transpires in this context. We conducted research utilising a porcine model due to the similarities in physiological mechanisms, metabolism, and the propensity for natural calcification in aortic valves when compared to humans. Additionally, we cultured three leaflets (cusps) of the aortic valve along with tissue and interstitial cells in vitro. Calcification was induced in aortic valve tissue and VICs in vitro through the incubation of osteogenic DMEM media for a duration of 21 days. Calcifications observed in the valves were predominantly composed of basic calcium phosphate crystals. A comprehensive series of experimental procedures was undertaken to achieve the objective. We conducted alizarin and von Kossa staining, yielding positive results in comparison to the control group. Quantitative reverse transcription polymerase chain reaction has provided the fold change values necessary for assessing the regulation of genes, including Twist1, Bmp2, OCN, OPN, and α-SMA. Finally, we conducted immunofluorescence staining to validate our hypothesis regarding dimerization. The findings indicate progress in the development of an inhibitor targeting the calcification process by obstructing dimer formation and inhibiting the activity of the specific protein involved in this mechanism.

Keywords: Aortic valve, Calcification disease, Markers of calcification, Aortic porcine model, valve interstitial cells, Dimerization, gene regulation, immunofluorescence

Contents

SL NO	TITLE	PAGE NO
1.	Acknowledgement	I
2.	List of Figures	II
3.	Abbreviations	III
4.	Abstract and Keywords	IV
5.	Introduction	1
6.	Review of Literature	3
7.	Materials and Methods	5
	➢ Porcine aortic valve collection	5
	➢ In Vitro cell culture and Valvular Interstitial cells isolation	6
	➢ Cell splitting method	6
	➢ Osteogenic Induction of the isolated valvular interstitial cells	6
	➢ Alizarin Red Staining	6-7
	➢ Von Kossa staining	7
	➢ RNA isolation using PureZOL reagent	7
	➢ RNA concentration measurement &cDNA preparation	7-8
	➢ RT-qPCR	9

	➢ Immunofluorescence Staining	10-11
8.	Results and Discussion	12
9.	Conclusion	21
10.	Appendix	22
11.	References	23
12.	Plagiarism Report	26

Introduction

The development of heart valves is marked by the epithelial to mesenchymal transition (EMT) of highly proliferative undifferentiated progenitor cells within the endocardial cushions, situated in a complex stratified extracellular matrix (ECM) in the mature valves. This transformation transpires in the fully developed valves. A reduction in the proliferation of progenitor cells, coupled with an enhancement in the complexity and organisation of the stratified extracellular matrix (ECM) in the semilunar (SL) and atrioventricular (AV) valve leaflets, is a defining feature of heart valve remodelling during foetal and neonatal development. [(1), (2)]. In the forthcoming decades, the burden of disease attributable to valvular heart disease is expected to escalate. Valvular heart disease is the primary cause of cardiovascular morbidity and mortality globally. Tricuspid valves, including the aortic valve, comprise 61% of all cases, whereas mitral valve illnesses constitute 15% of all cases. Global pathologies include rheumatic heart disease, aortic valve stenosis (characterised by elevated pressures behind the obstruction and inadequate pressures ahead), mitral regurgitation, and aortic regurgitation (which permits retrograde flow, leading to pressure equalisation that may compromise cardiovascular function). In the industrialised world, aortic valve stenotic disease is more common due to its known correlation with advanced age, obesity, high plasma lipoproteins, diabetes mellitus, and poor health circumstances (3). It is estimated that between 2% - 4% of persons over the age of 65 may acquire calcific aortic stenosis. 25% of people in this age group will present with symptoms of the condition, which results in a 50% greater risk of cardiovascular-related events. In addition, there is a chance of progression to heart failure, aortic valve replacement, or death that is related to this condition, which is 80% over five years. [(3), (4)].

For the purpose of our investigation, we have concentrated more on the calcification of the aortic valve by creating an in vitro culture environment with three leaflets of pig aortic valve. The thickening of valve leaflets is connected with chronic aortic valve disease (CAVD), which is triggered by many osteogenic signalling pathways. These pathways require the participation of several transcription factors, including Twist1 (a class II bHLH tissue-specific protein), Col2, and others. As a result, the osteoblastic differentiation in the valve is comparable to physiological osteogenesis, leading us to hypothesize that the Twist1 dimerization that occurs in bone calcification might also take place in the aortic valve. Craniosynostosis is a frequent ailment that is characterised by craniofacial deformities, skeletal anomalies, and limb patterning deficits (5). Saethre-Chotzen syndrome is a condition that occurs

when a mutation in the Twist1 gene (Twist1 haploinsufficiency) causes premature closure of the cranial suture. The Twist1 gene has been shown to include over fifty distinct mutations, all of which are projected to result in a loss of function. With an intervening mesenchyme, the cranial suture is made up of two osteogenic fronts that are in opposition to one another. While the Twist1 protein is expressed in the mesenchymal region and throughout the fronts, the Id1 protein, which is a class III transcription factor that contains the HLH protein, is only expressed in the fronts. The Tcf3 transcription factor was another class I transcription factor that coded for the E protein that was expressed in the front and the mid mesenchymal region. Id tends to bind with E protein in the osteogenic fronts and forcing Twist1 to homodimer (T/T) in the fronts and that leads to osteoblastic differentiation and formation of the bone and T/E heterodimers in the mid-suture mesenchyme induce TSP-1 expression, which then activates latent transforming growth factor-beta (TGF-β) and inhibits differentiation. There is a possibility that (TGF-β) activity and bone morphogenetic protein (BMP) work together to confine Id1 expression to the osteogenic fronts. Haploinsufficiency in the Twist1 gene causes an increase in the ratio of T/T to T/E, as well as conditions that encourage the creation of T/E in the suture, which ultimately leads to an undeveloped skull (6). Starting from this point, we have made an effort to demonstrate that the production of osteogenic protein factors also stimulates the formation of dimers and contributes to the process of calcification in the aortic valve.

Review of literature

The aortic valve is one of four heart valves that make sure blood flows only from the left ventricles to the aorta. The aortic valve has three thin collagenous leaflets that are linked to the fibrous ring at the end of the left ventricle. Each of them has three histological layers. The lamina fibrosa faces the aorta and is rich in collagen. The ventricularis faces the left ventricle and is rich in elastin. The intermediate layer is a gelatine layer called spongiosa that is rich in GAGs, proteoglycans, and other substances. Valvular interstitial cells (VICs) cover all of these extracellular layers, while valvular endothelial cells (VECs) safeguard the whole system. (7) During development, endothelial cells that cover the original valve-cushion go into the matrix below and change from endothelial cells to mesenchymal cells (EMT) (8). That's why VICs come from endothelial cells.

Calcific Aortic Valve disease gets worse over time. It starts with thickening of the aortic valve (sclerosis) and ends with valve stenosis and insufficiency later in life (9). It also causes fibrosis that causes ectopic mineralisation of AoV leaflets (cusps), which happens at the same time as osteogenic transitions of VICs, which causes microhaemorrhage of ECM. Collagen made by VICs acts as a scaffold for the beginning of calcium nucleation (10). CAVD can be caused by several things, such as metabolism, degeneration, inflammation, and some hereditary factors. Researchers looked at adult sick AoV to find indicators of valve development in order to find similarities between the disease's progression and the molecular processes that cause it (11). The process of aortic valve calcification begins with initial endothelial injury, which creates an access point for LDLs, monocytes, macrophages, and T cells. These get into the leaflets and cause long-term inflammation that leads to the propagation stage, which turns on the VICs. After that, metalloproteinases slowly reorganise the valvular matrix and release elastokines, which causes sclerosis, which doesn't cause any symptoms as you get older. Myofibroblasts cause the ECM to contract, which happens due to the increased level of α-SMA (marker of myofibroblastic cells), and when cells group on the reorganised matrix, they suffer apoptosis. This causes calcium salts to settle around the collagen chain and elastin in the valve tissue. [(12), (13), (14)]

Calcification mostly happens in the outer histological layer because endothelial lesions are more likely in the fibrosa. After all, they have a lot of collagen and elastin, which makes calcium bind and causes stenosis and blockage of blood flow. The size of a stenotic valve is 1 cm2, while the size of a normal valve is 2.5 cm2 to 4.5 cm2. (15)

It is thought that about 13% of calcified aortic valves at the time of surgery had real bone cells, called osteoblasts (which organise the calcium crystals in the matrix) and osteoclasts (which manage the tumour by resorption) (16). Runt-related transcription factor 2 (Runx2) and bone morphogenic protein (Bmp2) are two proteins that play a big role in osteoblastic development, which is what causes calcification. Osteoblasts release a lot of calcific proteins (markers) into bone tissue, including Osteocalcin (OCN), Osteopontin (OPN), and alkaline phosphatase (17). The molecular and cellular processes that control the change from proliferative, migratory, undifferentiated valve progenitor cells of the EC to the differentiated, relatively inactive VICs of the mature valves. (18). Valve progenitors grow and move around a lot and have transcription factors Twist1, Tbx20, and Msx1/2. Also, only in mature calcified AoV were there signs of active BMP signalling, such as the phosphorylation of Smad1/5/8 and the production of osteogenic genes, such as Runx2. The fact that these markers are expressed differently shows that an osteogenic regulatory mechanism is involved in CAVD in a specific way (19). Like heart valve and bone growth, investigations of human explanted valves show that BMP, Notch, and Wnt signalling pathways are involved in the evolution of CAVD (19). Klotho-/- mice, which are a model of accelerated ageing, show calcification that happens without inflammation or cusp thickening. This is the first evidence of a valve-intrinsic molecular basis for age-related calcification that is typical in older people. Runx2, Osteopontin, and Sox9, which are osteogenic factors, are more active in Klotho-/- AoV. In the Klotho-/- mice, calcification happens after Smad1/5/8 is activated more. Blocking BMP signalling is a promising new way to treat CAVD (20). Twist1 controls the growth of several growing organ systems, such as the heart, and also encourages EMT during development and when tumours spread (21). A study found that in human sick aortic valves, there was more Twist1 expression and cell growth, which led to more Tbx20 and extracellular matrix (ECM) gene expression next to nodules of calcification. Additionally, various experimental methods, including von Kossa and immunofluorescence staining on cultured and living human valves, have revealed that 5 out of 8 diseased valves exhibit Twist1-positive immunostaining. In general, sick human aortic valves have more Twist1 and Collagen 2 in places where the ECM is disorganised and where cells are dividing more quickly around calcified nodules(22).

Materials and Methods

- **Porcine aortic valve collection**

Porcine hearts were collected from a government pig slaughterhouse situated in Tangra, Kolkata. 1X PBS, forceps, scissors, dissection plate, and beakers were autoclaved the day before sample collection. The hearts were thoroughly washed with ice-cold autoclaved 1X PBS immediately after the slaughter to wash out the blood. The leaflets of the aortic valve, which is situated at the base of the aorta, were isolated using sterile forceps and scissors. With this process, we had to keep on pouring 1X PBS into the heart valves to make sure there was no blood left in the leaflets. After collecting each leaflet, they were kept in the chilled 1X PBS in a small beaker to wash out any remaining blood before culture.

- **In Vitro cell culture and Valvular Interstitial cells isolation**

1. The three aortic valve leaflets(tissues) were taken out, and for the detachment of the cells a sterile 35mm plate with 3ml of cold collagenase solution (DMEM+collagenase).
2. The leaflets were placed into the plate filled with collagenase solution and incubated for 5-10 minutes at 37°C and 5% CO_2.
3. Valvular endothelial cells (VECs) were removed by rotating a dry sterile swab onto the surface of the leaflets.
4. A sterile 15 mL Falcon was filled with 3 mL of collagenase solution per leaflet.
5. After swabbing the VEC layer immediately placed in the Falcon filled with Collagenase solution and incubated for 12-18 hours. (23)
6. Then the degraded tissues are mixed with a serological pipette until the collagenase solution homogenizes the tissue, which helps in breaking up the tissue and releasing the VICs.
7. Centrifuged the digested tissue for 5 mins at 1000 rpm and aspirated the supernatant.

8. 5 ml of complete medium (DMEM + FBS) was added to a 15 ml falcon and centrifuged for the second time, followed by aspirating the supernatant.
9. Resuspended the interstitial cells in 5 ml of complete medium.
10. The cells were plated on one side of the T-75 flasks that were previously collagen-coated [for coating, we had used NaOH,1X PBS, collagen mixed solution transferred to the flasks and incubated for a minimum of 2 hours. Before plating the cells, the T-75 flask was washed with 1X PBS and let them grow for 1-2 days before it's time to split the cells into more culture flasks.

- **Cell splitting method**

1. The spent media was aspirated from the flasks and gently washed the cells with 1X PBS, rocked and discarded.
2. Trypsin-EDTA was added on the side where the cells are harvested and incubated at 37°C and 5% CO_2 for minimum 5-6 min until the 90% cells get detached from the surface.
3. Complete media was added to the flasks and flashes several times on the surface by pipetting.
4. After that, all the cells were transferred to a falcon and centrifuged at 1000 rpm for 5 minutes.
5. Discarded the supernatant and resuspended the cell with complete media.
6. Then the cells were plated in the previously collagen-coated flasks.

- **Osteogenic Induction to the isolated valvular interstitial cells**

The calcification was induced in the cells using 10mM osteogenic (OS) media that contains complete media with glycerophosphate, glycerophosphate-ascorbic acid, and dexamethasone and incubated at 37°C and 5% CO_2 for 21 days. Dexamethasone is very light sensitive so we used to cover up the bottle containing OS media with aluminium foil all through. The media change took place every two days.

- **Alizarin Red Staining**

Alizarin red is a commonly used stain to identify calcium-containing osteocytes in differentiated cultures of both human and mesenchymal stem cells where calcium forms alizarin red-s calcium complex in a chelation

process and the end product is a bright red stain. Here we had used the stain to detect calcium deposition in the cells in VICs. The method we had followed was,

1. First the media was removed from a 35 mm plate and washed thoroughly with 1X PBS twice for 5 mins.
2. Then fixed the cells and the tissue with 4% PFA (paraformaldehyde) for 10 mins in a rotating shaker.
3. After that wash the cells and tissues with 1X PBS for twice and distil water once.
4. 2% alizarin red solution (alizarin powder + do) was added to the plates and kept in the shaker for 10 mins.
5. The solution was discarded, and the plates were washed with dH_2O to destain until the stain and cell debris were removed from the surface of the cells other than the calcium deposited site. Then it's ready to visualize under a microscope.

- **Von Kossa staining**

The Von Kossa histological stain is widely used to quantify mineralization in cell culture and histological sections. We here used to see the presence of abnormal calcium deposits in the cells. The stain detects phosphate salt in cells using silver nitrate; calcium ions bound to phosphate are replaced by silver ions. Counterstaining the nuclear red allows for to staining nuclei of the cells in red and cytoplasm in pink. Calcium deposited site stains dark pink to blackish.

The method followed was-

1. First, discard the media from the 35 mm plate and washed with 1X PBS.
2. Added 10% formalin and kept in a shaker for 20 minutes.
3. The plate was Washed, the formalin was added cells and washed with dH_2O twice.
4. 1% silver nitrate solution was added and kept in bright light exposure with the lid open for 45 mins.
5. Checked whether the cells were coloured brownish, then washed the silver nitrate solution with milli q water.
6. Next 2.5% sodium thiosulfate solution was added and kept it for 5 mins in the shaker.
7. It was washed with dH_2O.

8. For the counterstain nuclear fast red was used. 5% aluminium sulfate solution was added in a falcon and kept it in a beaker having boiled water , then added 0.1% nuclear fast stain to the boiled aluminium sulfate solution until it get dissolved.
9. Cooled down the stain solution and filtered it with a syringe filter then added to the plate. Keep it for 5 mins.
10. Finally washed with dH_2O until destain the dark pink stain and then visualized under a microscope.

- **RNA isolation using PureZOL reagent**

1. The spent media was discarded from the plates where the cells were cultured and 1ml of PureZOL was added to the plates and incubated the lysate at room temperature for 5 mins, to allow the complete dissociation complex.
2. 0.2 ml of chloroform was added to the PureZOL solution and shaken vigorously for 15 secs and incubated for 5 mins.
3. Then centrifuged at 12000 rpm for 15 mins at 4℃.
4. Without disturbing the interphase, immediately transferred the aqueous phase to a new tube.
5. After that 500 µl isopropanol was added and incubated for 5 mins at room temperature. Again, centrifuged at 12000 rpm for 10 mins at 4°C.
6. The pellet that contained RNA washed with 70% ethanol and centrifuged for the last time at 7500 rpm for 5 mins at 4°C.
7. Resuspended the pellet in DEPC water (RNAse-free water) and stored it at -80°C.

- **RNA concentration measurement**

RNA concentration was measured by a UV-Vis spectrophotometer.
Before the OD measurement, the sample was diluted by DEPC and measured at A/260 and A/280 wavelengths. The concentrations of all the samples were calculated by the Lambert-Beer law ($A= \varepsilon.c.l$).

- **cDNA preparation**

For qRT-PCR, cDNA was made from the isolated RNA using a gradient PCR machine.

cDNA preparation was included - Reaction mix-10 µl, Reverse Transcriptase: 2 µl, and dH_2O (8 - RNA)

RNA of all the samples (calculated according to 2µg amount) was isolated and kept in the PCR tubes. These mixes were run on gradient PCR with the protocol used-

1. Incubated the reaction mixture at 25°C for 5 mins.
2. Incubated at 42°C to 55°C for 30 mins.
3. Inactivated the reaction by heating at 95°C for 1 min.

- **Real Time Quantitative Reverse Transcriptase PCR (qRT-PCR)**

qRT-PCR has been done to identify and understand the molecular mechanisms of calcification. To recognize the transcription factors, the markers that are responsible for it, and the downregulation-upregulation of those genes through Ct value and fold change. This technique has given us proof of those genes we thought were responsible and the alteration to them.

1. The qRT of the samples was done with several genes of interest that are the markers of osteogenesis, including Bmp2, OCN, OPN. The housekeeping gene was chosen actin.
2. The reaction mix was contained- Sybr green, DEPC, Forward and Reverse primer for the specific gene (which were previously standardised), cDNA. The amount of all these were calculated according to the number of sample usage.
3. The process we had followed - a. First two reaction mixes were made in 1.5 ml tubes. One was for the gene of interest and another one was for the housekeeping gene. Tubes must be kept in a mini ice tray in a dark place.

b. Then 27 µl from the mix was kept in each pcr tube allotted for the samples and in to that added 3 µl of cDNA.

c. The reaction mixes were given to each well allotted for genes of interest and housekeeping genes of the qRT plate and covered with micro seal properly and made sure that no evaporation of the sample takes place. For each sample there were three wells for technical replication of three and to statistically prove the obtained data set and run the experiment with the proper annealing temperature that was standardized previously for primer and settings so that machine can read the plate and give the data.

- ## Immunofluorescence Staining (IFS)

Immunofluorescence technique is a widely used immunochemical technique that allows detection and localization of target antigen in any tissue or cell culture and utilizing their binding specificity to fluorophore tagged antibodies given to identify the deposition of abnormal proteins and molecules in the tissue or cells. It's the significant example of immunostaining that uses antibodies to stain the proteins and immunohistochemistry (the use of the antigen-antibody relationship in tissues). Fluorophore-tagged antibody visualizes the position of biomolecules of interest by exciting the fluorophore to a certain wavelength and measures the emission of light in a specific predefined wavelength using fluorescence microscopy. It also provides us with unprecedented insights into human health and disease. Many different types of IFS are there, here we had done IF double staining firstly to detect the levels and locations of Twist1 and α-SMA (activates fibrogenesis in cells) proteins in the cultured cell for both control and OS treatment and also to visualize the dimer nature of the Twist1 protein using E protein and Id1 protein. This staining had given reliable data about the Twist1 dimerization and function in the VICs that was useful for further design of the rest of the experiments. To remember double staining we must use two antibodies derived from two different animals (generally mouse and rabbit) to get different colours of the different proteins.

The protocol we followed for the double IF staining is -

1. First the spent media was discarded from the plate and washed with 1X PBS twice for 5 mins.
2. 4% PFA was added to fix the metabolism for 10 minutes. Washed cells with 1X PBS after discarding the PFA.
3. Then a freshly made permeabilizing buffer containing 0.2% Triton in 1X PBS was added and kept in the shaker for 10 mins.
4. The excess buffer was washed with ice-cold 1X PBS twice for 5 minutes.
5. Rinsed with the freshly made washing buffer containing 1X PBS+0.05% tween20 was given once for 1-2 mins.
6. Blocking buffer [2% BSA (bovine serum albumin) in PBST (T stands for triton or tween 20, either can be used) was added to the plate and kept it for 1 hour.
7. After 1 hour, cells were rinsed with wash buffer containing 1X PBS and 0.05% Tween 20 for 5 minutes to wash out excess BSA, ensuring that antibodies don't encounter any issues binding to the protein.
8. The primary antibody, which was dissolved in the blocking buffer of the first protein, was added to the plate and incubated at 4°C overnight on a shaker.
9. **On the very next day**, washed with 1X PBS thrice for 5 mins and added the fluorophore tagged secondary antibody (used of mouse that emitted fluorescence

light at 555 nm)that was dissolved in PBS and kept it for 2 hours in shaker while covered up to prevent light as the fluorophore are there.

10. **For double staining**, after the 1X PBS washout the excess antibodies were thrice for 5 mins of blocking buffer was added and kept it for 1 hour.
11. Washed with 1X PBS and given the primary antibody dissolved in PBS of the second protein overnight at 4°C in a shaker.
12. The next day washed with 1X PBS and added a secondary antibody of rabbit that emitted light at 488 nm for 2 hours while covered up in a shaker.
13. Washed with PBS thrice and added DAPI dissolved in PBS for 1 hour that stains the nucleus and emits fluorescence at 365 nm.
14. Excess DAPI was washed with PBS and finally added mounting media containing impropyl that prevent quenching and glycerol that prevent the degradation of antibody. The plate is ready for visualizing under fluorescence microscope.

Results and Discussion

1. Porcine heart

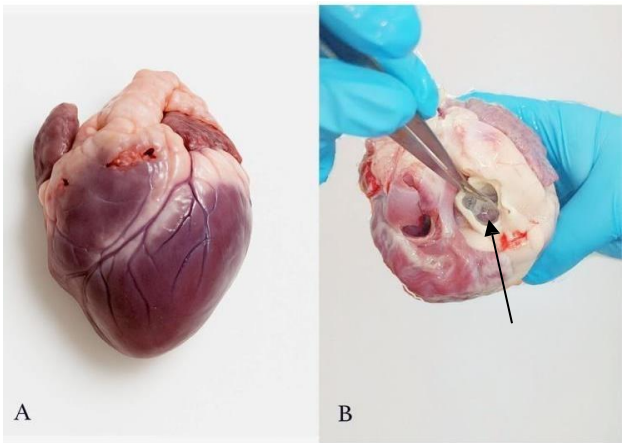


Figure1: Porcine whole heart(A) and the three leaflets of the aortic valve that are held with forceps are shown with an arrow(B)

2. Histological Analysis

A histological comparison between calcified interstitial cells and control cells of the aortic valve.

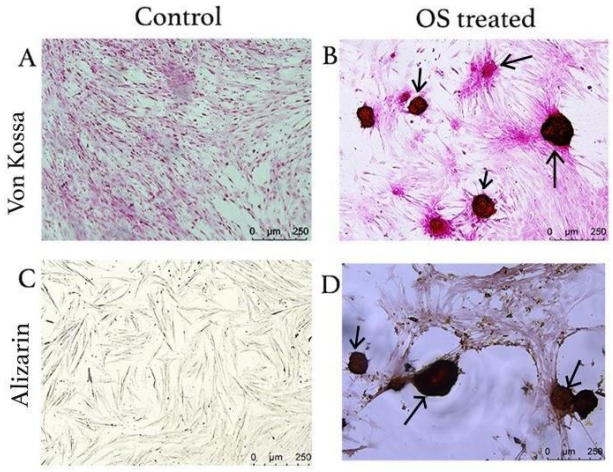


Figure2: Von Kossa staining result: control cells(A) are von kossa negative and the 21 days OS treated cells(B) are von kossa positive, where calcium

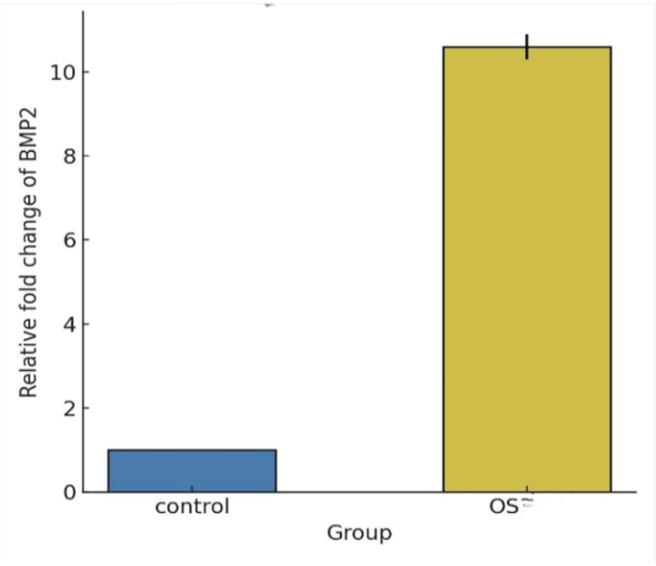
deposition sites(arrows) are showing dark pink to blackish colour and cytoplasm stains pink. Alizarin staining results: control cells(C) are alizarin negatives, and after 21 days of OS treatment, the positive are bright red cells(D) are showing a bright red colour where the calcium is deposited (shown with arrows). All the pictures are taken in 10X magnification under a microscope, and the scale bar indicates 250m.

Discussion: Alizarin and Von Kossa both stains have given the histological evidence of the calcification in the valvular interstitial cells of the porcine heart. From the fig 2 it is shown that after 21 days of osteogenic treatment, most of the cells (B&D) are aggregates in the ECM, and calcium deposition has taken place in the outer histological layer though that couldn't be understood through it. Alizarin red S stain only bound with the calcium salt in the cells so it took a bright red colour in the calcified region. While in von Kossa staining where silver nitrate was used which detects the phosphate salt with which calcium binds and is replaced by silver nitrate when exposed to light, and appears as black deposits under a microscope. With the counterstain, the calcific area stains blackish pink, and the nucleus is stained red, and the cytoplasm pink. So both the stains indicate that those arrowed os treated regions are the calcified region of the aortic valvular interstitial cells.

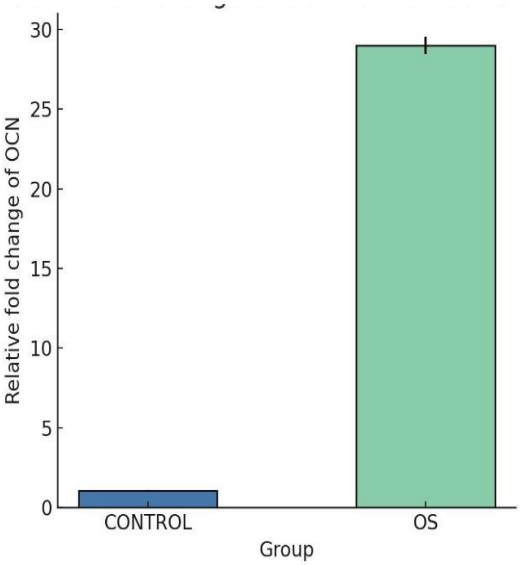
3. Molecular analysis

There are three specific markers of our interest, the upregulation of which indicates calcification in the cells of the aortic valve. Real-time quantitative PCR has made it easy to analyse this molecular aspect of CAVD.

A.



B.



C.

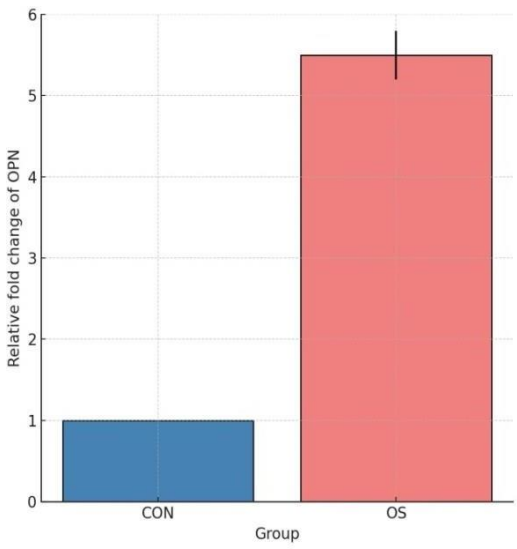
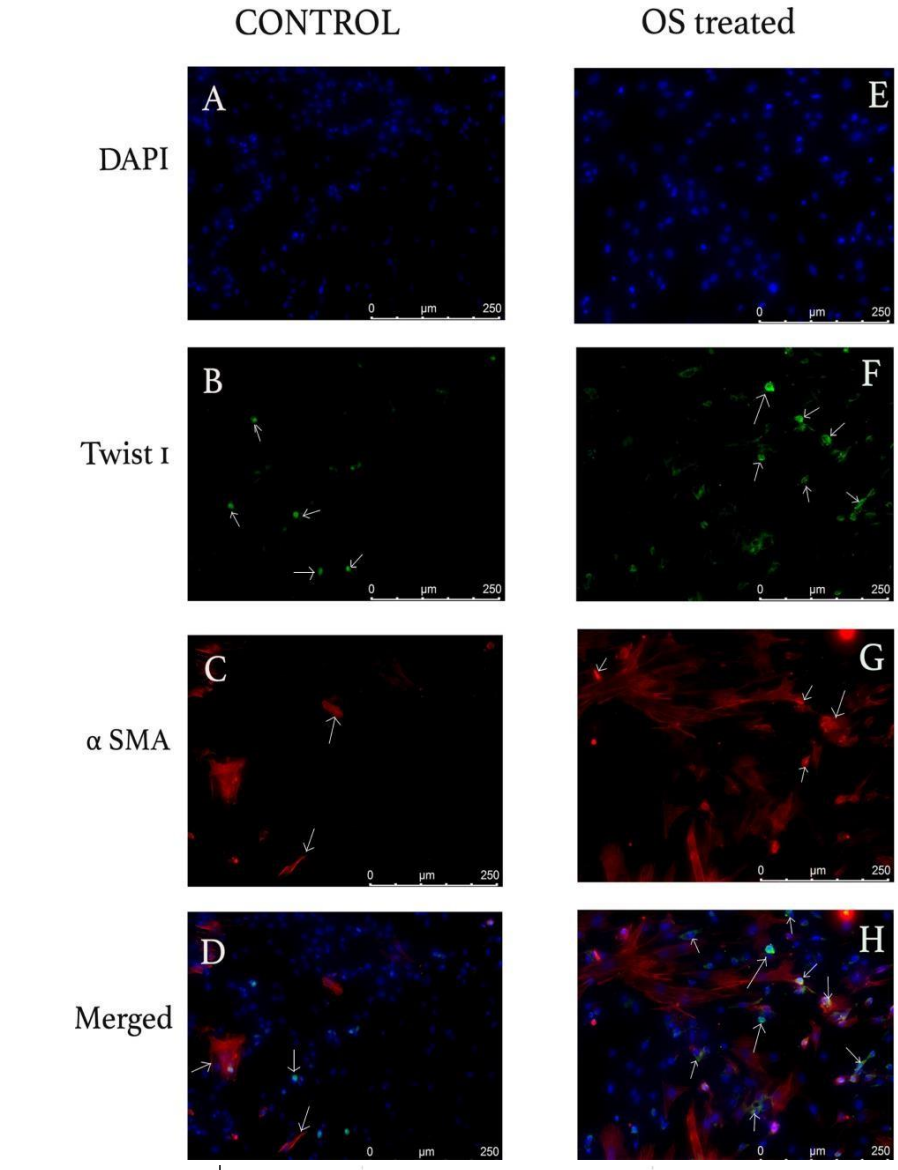


Figure3: qRT-PCR analysis of mRNA shows expression of Bmp2(A), OCN(B), and OPN(C) is increased significantly after 21 days of osteogenic treatment condition compared to control. This graphical data suggests successful calcification induced in the interstitial cells of the aortic valve.(n=2)

Discussion: Isolated valvular interstitial cells from the porcine model are subjected to induce calcification by the OS media for 21 days. This qRT-PCR analysis shows a 9.91-fold increase of Bmp2 marker (Fig.3A), a 30.27-fold increase of OCN marker (Fig.3B) and a 5.54-fold increase of OPN marker (Fig.3C), which indicates that the valve calcification takes place because of the upregulation of these markers. Significant upregulation of the Bmp2 marker assures that the Bmp2/smad pathway, which plays an important role in osteoblastic differentiation and calcification, has taken place in the valve that follows the upregulated expression of OCN and OPN markers. And with the increased expression of Bmp2, Id1 protein level will also increase as the α SMA So, in the molecular level, these osteoblastic markers, which were thought to be as they are bone markers in developing bone, are also shown to be significant in causing CAVD.

4. Immunofluorescence analysis

Immunofluorescence staining has significantly helped to ascertain the abundance of the markers thought to be responsible for the calcification in the aortic valve. Also, to get the evidence about dimerisation had to resort to this method.



I.

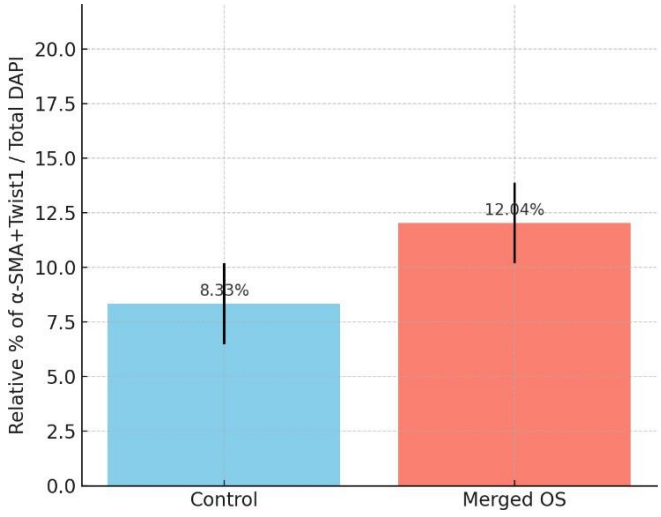
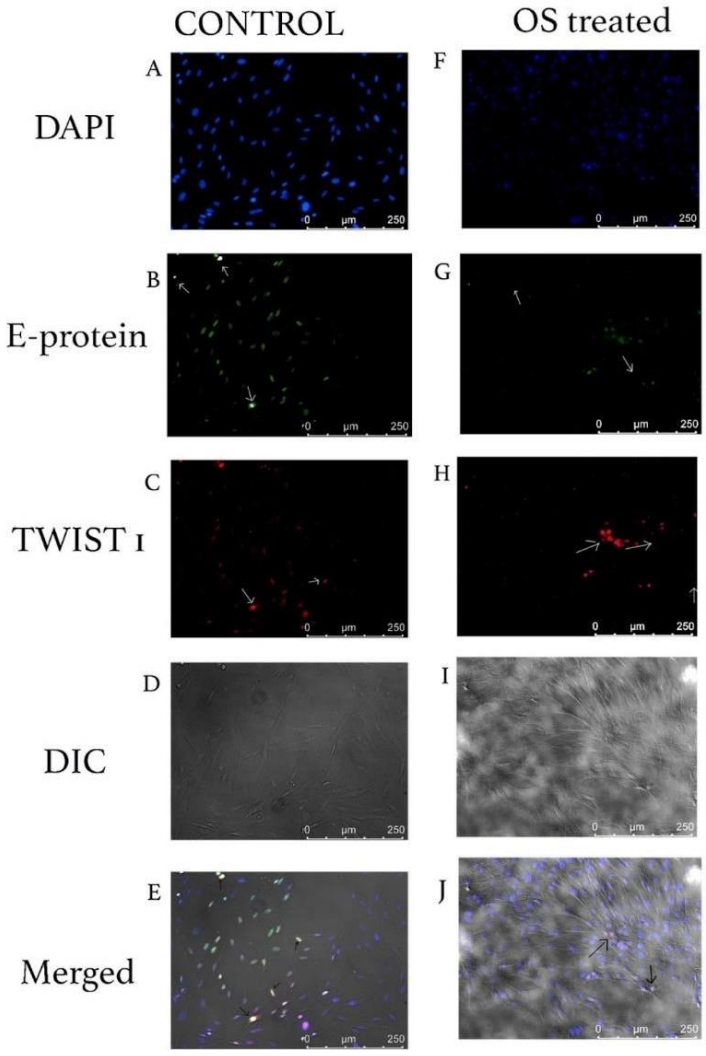


Figure4: Double IF staining of Twist1 and α-SMA marker protein: In control cells (A-D) abundance of Twist1(in green, B) and α SMA (in red, C)

are showing low. But in 21 days of OS treated cells (E-H), both Twist1(F) and α SMA (G) are abundant that are more specifically in the merged image(H). Twist1 is nucleus-specific so it the stained there only and expressed as a dot structure like DAPI (B, F) but α SMA is cytoplasmic-specific specific so the staining is expressed throughout the cell in a wide range(C,G). The cell surface can easily be detected (arrowed region). [scale bar indicates 250 m, magnification - 10X]. And the graphical representation of the relative % of total number of twist1+α SMA positive cells and total number of DAPI positive cells in OS in comparison to control (I).

Discussion: Twist1 and α SMA staining is done to prove the presence of twist1 and myofibroblastic cells in the aortic valve. α SMA denotes activated fibroblast. Typically, abundant α SMA activates fibrogenesis in the valve, resulting in thickening of the valve leaflets termed as sclerosis. Fibroblastic cells are the major cause of calcification and narrowing of the passage of the aortic valve and cause CAVD. In figure4 control cells and the os treated cells are distinguishable by the expression of these proteins. As twist1 is nucleus specific, it's chosen to add a fluorophore that emits light at 633 nm that can only be detected by the microscopic camera. Also, α SMA is cytoplasmic specific so the fluorophore was chosen that emits light at 488 nm. In unaffected regions of the diseased valve twist1 and α SMA are not detected. A relative percentage of twist1 + α SMA positive cells graph (Fig. 4I) has shown a higher percentage of positive cells in the OS treated cells than control cells. And from qRT-PCR, it's also seen that Bmp2 has upregulated So, it's proved that the partial major cause for calcification in the aortic valve is the increased level of twist1 and αSMA from the arrowed region shown in picture H.



K.

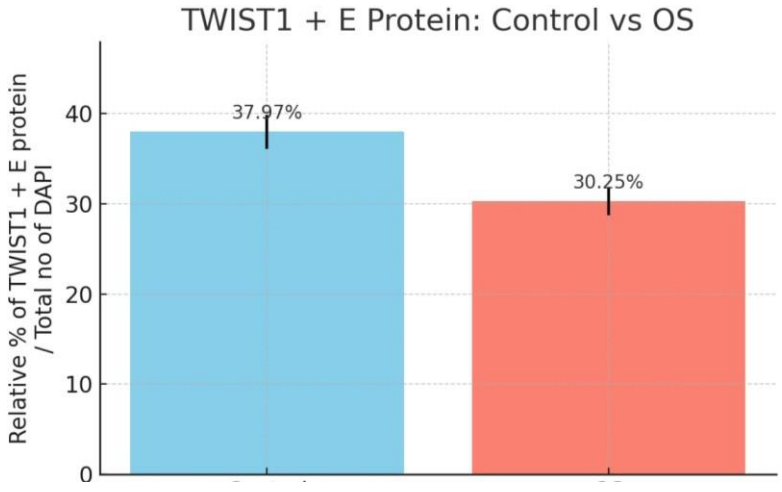
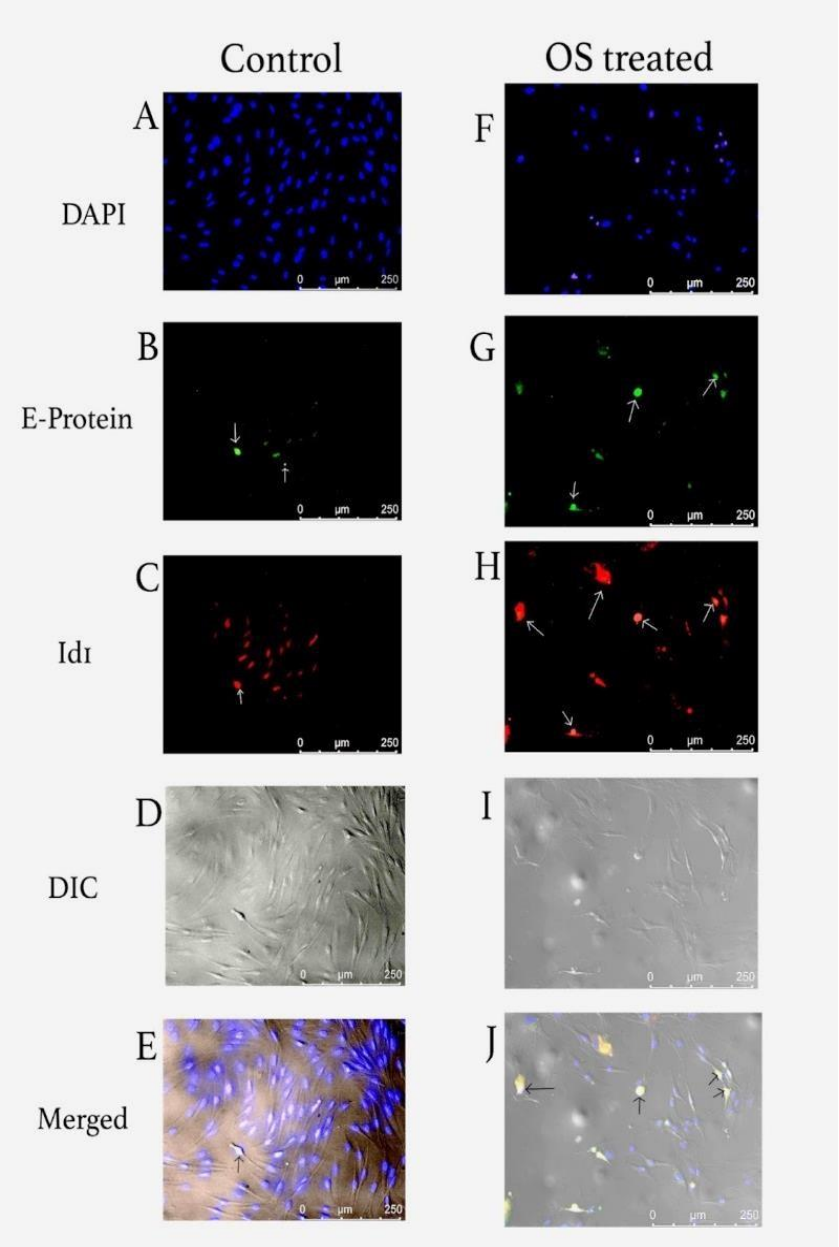


Figure5: Double IF staining of E and Twist1 protein: this staining is done to specify dimerization between Twist1 and E protein. Here in the control cells E

protein is shown in green(B) and twist1 in red(C) and in the merged picture(E), the
bright coloured regions where it's clearly showing red and green coloured are merged(arrowed regions) that express the T/E dimer. In the case of 21 days of os treated cells, there is very less or no T/E region as it shown in the picture(J). DIC images(H) are given for both cases because both the proteins are nucleus-specific, and to understand the cell's surface and the localization of the proteins in a bright field with high resolution. [scale bar indicates 250 m, magnification - 10X]. And the graphical representation of relative % of total number of twist1+ E protein positive cells and total number of DAPI positive cells in OS compared to control (K).



K.

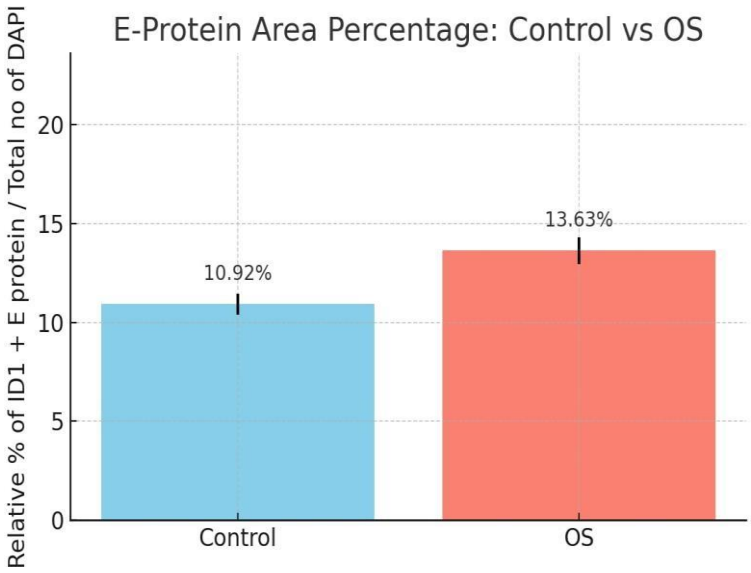


Figure 6: Double IF staining of E and Id1 protein: this staining is done to specify the dimerisation between E and Id1. Here in the control cells, E protein is shown in green(B) and Id1 in red(C), and in the merged picture(E), there are no such regions where both red and green colour got merged(E). But in the case of 21 days of OS treated cells, there are plenty of regions where the bright colour indicates merged red and green colour, other than the DAPI (blue colour, J), the regions are shown with arrows. DIC images(H) are given for both cases because both proteins are nucleus-specific, and to understand the cell's surface and the localisation of the proteins in a bright field with high resolution. [scale bar indicates 250 m, magnification - 10X].And the graphical representation of relative % of total number of Id1+ E protein-positive cells and total number of DAPI positive cells in OS compared to control (K).(n=2)

Discussion: As the hypothesis was made that the same dimerization takes place in the calcified aortic valve which takes place during skull development in cranial suture as because calcium is the building block for the skull bone development, this IF staining has assured the hypothesis that is seen in both the merged images of Fig 5 and Fig 6. In the control cells, according to the hypothesis it's seen the dimer formation between Twist & E protein heterodimer is significantly increased (fig5),

but not the dimer between Id1 & E protein (Fig6). The graphical analysis of the Twist1 + E protein positive cells (Fig.5K) also shows a higher percentage of positive cells present in the control cells than OS treated cells. While in the os treated cells, the scene gets reversed. When the calcification is induced in the interstitial cells, as the hypothesis has said, Id1 protein tends to bind with E protein and let twist1 to form a homodimer it was the target to get assurance about this through this IF staining (Fig6). And it is seen that the greater number of Id1 bound to E protein expressed as the bright red and green mixed colour (H), indicating the positive staining that has been aimed for. Along with this, the graph also is showing the percentage of Id1 + E protein-positive cells is higher in the OS treated cells than control. So, it is evident that in the calcific aortic valve, twist1/twist1 homodimer forms.

Conclusion

Cardiovascular diseases (CVDs) pose a significant global threat, affecting over 23.6 million people by 2030. Adult cardiac diseases are often linked to structural and functional insufficiency of adult aortic valves, leading to aortic cusp mineralisation, calcification, and dysfunction. This thesis has elucidated the multifaceted role of Twist1 in the pathogenesis of calcific aortic valve disease (CAVD), highlighting its dual functions in valve development and disease progression. This research was focused on understanding its induction and progression. From the several methods used to reach the aim, it can be concluded that there is a direct connection between physiological bone development(osteogenesis) and the CAVD because most of the bone markers that are responsible for normal bone growth are present at a significantly high level in the diseased valve. The quantitative analysis of the markers, IF staining, has given evidence about the presence and abundance of the osteoblastic markers twist1, bmp2, OCN, OPN, α SMA, Id1, etc. Twist1 is always abundant in the calcific valve, likewise, the other bone markers are there at high levels, and also the dimer formation has an effect on the calcification. surgical interventions for CAVD are limited due to thrombogenicity or degeneration complications, and repairing existing valves is more effective than valve replacement. Human models can also be used to get the experiments done, but it's difficult to get because there has to go through a lot of procedures, including permission to collect the heart from a hospital. That's why it's much easier to work on such models as porcine, mice, etc. So, for further study, it is needed to get strong proof for the dimerization that has been established through IF staining by CO-Immunoprecipitation in downstream and inhibit the calcification by obstructing the twist1 homodimer formation by decreasing the level of Id1, along with that to inhibit the expression of the protein factors responsible for CAVD and downregulate them. Several inhibitors work at a post-transcriptional level, where they prevent the transcription factors, and those that work at the post-translational level affect proteins present in the diseased valve. It can be done to check if inhibitors can reverse the calcification. Harmine can be used for Twist1 inhibition, and cannabidiol for Id1 inhibition. In adult aortic valve calcification disease, the results of the present study have elevated the novel implications for anti-calcific therapeutics in heart valve clinics.

Appendix

Phosphate buffer saline (PBS)

1X PBS was made to use during heart-washing and for staining purposes.

a. To make 1000 ml 1X PBS, the reagents were 8 g NaCl, 0.2 gm KCl,1.44 g Na2HPO4, and 0.245 g KH_2PO_4 measured in a bottle.

b. In the bottle, 960 ml MilliQ water was added, properly dissolved by magnetic stirrer, and the pH was measured to be 7.38 by using HCl or NaOH, and the rest 40 mL of water and cross-checked the pH.

c. Autoclaved the PBS and used it purposely.

B. Dulbecco's Modified Eagle Media (DMEM)

It is the medium that was used to culture the valvular interstitial cells throughout the experiments. To make 1 L of media, the steps followed were,

Step 1: Penicillin-Streptomycin solution making - 50 ml of solution was made with distilled water and the pen-strep powder.

Step 2: 500 mL autoclaved distilled water was poured into an autoclaved conical flask, and DMEM powder was added to it and shaken well to dissolve properly. It was orange in colour.

Step 3: 3.7 g sodium bicarbonate was added to the solution to adjust the pH of the media, and the media colour changed to dark red. Then the rest 500 ml of distilled water was added and shaken well.

Step 4: 20 ml of media was removed from the conical and added 20 ml of pen-strep was added in its place of it.

Step 5: Then for the filter procedure the previously autoclaved glass filter equipment was used. Filter paper was used to filter the media, and a vacuum pump machine vacuum was created to help through the process. Must check whether the media gets leaked.

Step 6: At the end, 1 ml of antimycotic solution was added to the media by removing 1 ml of media to prevent fungal contamination. Stored the media in two 500 ml bottles a 4°C fridge.

C. Complete Media (growth medium)

Complete media was made with DMEM and FBS, that was used for culturing and growth of the cells. For making 50 ml of the media,

a. 5 ml of 10% FBS was added to a 50 ml Falcon and 45 ml of the DMEM media was added using sterile pipette.

b. Filtered the media using a 50 ml syringe and a filter in a Falcon and stored it in a 4°C fridge.

D. Osteogenic Media

Osteogenic media was used at a 10mM concentration to induce calcification in the cells. The components were added to the complete media. The processes were,

a. Firstly, 50 ml complete media was prepared with 45 ml DMEM and 5 ml of 10% FBS in a Falcon.

b. Then 5ml media was removed and 2.5 ml of glycerophosphate, 2.5 ml of L-ascorbic acid in place of it, and filter the media.

c. After that, 5 µL of dexamethasone was added to the Falcon with kept the light off and storing the media at 4°C fridge, which was covered with aluminium foil.

Reference

1. Chakraborty S, Wirrig EE, Hinton RB, Merrill WH, Spicer DB, Yutzey KE. Twist1 promotes heart valve cell proliferation and extracellular matrix gene expression during development in vivo and is expressed in human diseased aortic valves. Dev Biol. 2010 Nov 1;347(1):167-79. doi: 10.1016/j.ydbio.2010.08.021. Epub 2010 Sep 6. PMID: 20804746; PMCID: PMC2950232.

2. Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, Schoen FJ. Human semilunar cardiac valve remodeling by activated cells from fetus to adult. Circulation. 2006; 113:1344–1352. doi:10.1161/CIRCULATIONAHA.105.591768.[DOI] [PubMed] [Google Scholar]

3. Aluru JS, Barsouk A, Saginala K, Rawla P, Barsouk A. Valvular Heart Disease Epidemiology. Med Sci (Basel). 2022 Jun 15;10(2):32. doi: 10.3390/medsci10020032. PMID: 35736352; PMCID: PMC9228968.

4. Otto CM, Burwash IG, Leggett ME et al. Prospective study of asymptomatic valvular aortic stenosis. Clinical, echocardiographic, and exercise predictors of outcome, Circulation. 1997; 95:2262–70. doi: 10.1161/01.cir.95.9.2262.

5. Alushi B, Curini L, Christopher MR, Grubitsch H, Landmesser U, Amedei A, Lauten A. Calcific Aortic Valve Disease-Natural History and Future Therapeutic Strategies. Front Pharmacol. 2020 May 13; 11:685. doi: 10.3389/fphar.2020.00685. PMID: 32477143; PMCID: PMC7237871**.**

6. Connerney J, Andreeva V, Leshem Y, Muentener C, Mercado MA, Spicer DB. Twist1 dimer selection regulates cranial suture patterning and fusion. Dev Dyn. 2006 May;235(5):1345-57. doi: 10.1002/dvdy.20717. Erratum in: Dev Dyn. 2012 Feb;241(2):433. PMID: 16502419**.**

7. Lange FJ, Moorman AF, Anderson RH et al. Lineage and morphogenetic analysis of the cardiac valves, Circ Res. 2004;95:645 54. doi: 10.1161/01.RES.0000141429. 13560.cb. [DOI] [PubMed] [Google Scholar

8. Aikawa et al., 2006; Hinton et al., 2006**.**

9. Otto CM. Valvular aortic stenosis: disease severity and timing of intervention. J Am Coll Cardiol. 2006; 47:2141–51.

10. Lindman BR, Clavel MA, Mathieu P, Iung B, Lancellotti P, Otto CM, Pibarot P. Calcific aortic stenosis. Nat Rev Dis Primers. 2016 Mar 3; 2:16006. doi: 10.1038/nrdp.2016.6. PMID: 27188578; PMCID: PMC5127286.

11. Wirrig EE, Hinton RB, Yutzey KE. Differential expression of cartilage and bone-related proteins in pediatric and adult diseased aortic valves. J Mol Cell Cardiol. 2011; 50:561–9.

12.Feldmann A, Nitschke Y, Linß F, Mulac D, Stücker S, Bertrand J, Buers I, Langer K, Rutsch F. Improved Reversion of Calcifications in Porcine Aortic Heart Valves Using Elastin-Targeted Nanoparticles. Int J Mol Sci. 2023 Nov 17;24(22):16471. doi: 10.3390/ijms242216471. PMID: 38003660; PMCID: PMC10671589.

13. Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. Circulation. 1994; 90:844–53.

14. Farzaneh-Far A, Proudfoot D, Shanahan C, Weissberg PL. Vascular and valvar calcification: recent advances. *Heart*. 2001; 85:13–17

15. Zigelman CZ, Edelstein PM. Aortic valve stenosis. *Anaesthesia Clin*. 2009; 27:519–532.

16. Mohler ER III. Mechanisms of aortic valve calcification. *Am J Cardiol*. 2004; 94:1396–1402, A1396.

17. Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: methods, models, and mechanisms. Circ Res. 2011 May 27;108(11):1392-412. doi: 10.1161/CIRCRESAHA.110.234138. PMID: 21617136; PMCID: PMC3150727.
18. Combs MD, Yutzey KE. Heart valve development: regulatory networks in development and disease. Circ Res. 2009 Aug 28;105(5):408-21. doi: 10.1161/CIRCRESAHA.109.201566. PMID: 19713546; PMCID: PMC2777683.
19. Gomez Stallons MV, Wirrig-Schwendeman EE, Fang M, et al. Molecular Mechanisms of Heart Valve Development and Disease. 2016 Jun 25. In: Nakanishi T, Markwald RR, Baldwin HS, et al., editors. Etiology and Morphogenesis of Congenital Heart Disease: From Gene Function and Cellular Interaction to Morphology [Internet]. Tokyo: Springer; 2016. Chapter 18. Available from: https://www.ncbi.nlm.nih.gov/books/NBK500307/ doi: 10.1007/978-4-431-54628-3_18
20. Cheek JD, Wirrig EE, Alfieri CM, James JF, Yutzey KE. Differential activation of valvulogenic, chondrogenic, and osteogenic pathways in mouse models of myxomatous and calcific aortic valve disease. J Mol Cell Cardiol. 2012; 52:689–700.
21. Barnes and Firulli, 2009; Yang et al.,2004
22. Chakraborty S, Wirrig EE, Hinton RB, Merrill WH, Spicer DB, Yutzey KE. Twist1 promotes heart valve cell proliferation and extracellular matrix gene expression during development in vivo and is expressed in human diseased aortic valves. Dev Biol. 2010 Nov 1;347(1):167-79. doi: 10.1016/j.ydbio.2010.08.021. Epub 2010 Sep 6. PMID: 20804746; PMCID: PMC2950232.
23. Cuevas RA, Chu CC, Moorhead WJ 3rd, Wong R, Sultan I, St Hilaire C. Isolation of Human Primary Valve Cells for In vitro Disease Modelling. J Vis Exp. 2021 Apr 16;(170):10.3791/62439. doi: 10.3791/62439. PMID: 33938898; PMCID: PMC8406428.

Somdutta Bhattacharyya

Revised_Somdutta Bhattacharyya

Dissertation report
Dissertation projects 2025
Presidency University, Kolkata

Document Details

Submission ID
trn:oid:::1:3264254265

Submission Date
May 30, 2025, 1:58 PM GMT+5:30

Download Date
May 30, 2025, 2:02 PM GMT+5:30

File Name
Revised_Somdutta_Bhattacharyya.pdf

File Size
920.3 KB

23 Pages
6,632 Words
33,788 Characters

5% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

Filtered from the Report

- Bibliography
- Quoted Text

Match Groups

29 Not Cited or Quoted 5%
Matches with neither in-text citation nor quotation marks

1 Missing Quotations 0%
Matches that are still very similar to source material

0 Missing Citation 0%
Matches that have quotation marks, but no in-text citation

0 Cited and Quoted 0%
Matches with in-text citation present, but no quotation marks

Top Sources

4%	Internet sources
3%	Publications
2%	Submitted works (Student Papers)

Integrity Flags

0 Integrity Flags for Review

No suspicious text manipulations found.

Our system's algorithms look deeply at a document for any inconsistencies that would set it apart from a normal submission. If we notice something strange, we flag it for you to review.

A Flag is not necessarily an indicator of a problem. However, we'd recommend you focus your attention there for further review.